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Effect of chemical cationization of antigen on glomerular localization of immune complexes in active models of serum sickness nephritis in rabbits

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SUMMARY

The effect of chemical cationization of antigen on the glomerular localization and formation of immune complexes (IC) was investigated utilizing the models of acute accelerated and chronic serum sickness nephritis in rabbits. In acute accelerated serum sickness, neither antibody nor antigen was detected in the glomerulus before the second injection of antigen. At 15 min after the challenge, rabbits given cationized BSA developed IC deposition along the peripheral capillary walls, whereas no IC deposition was found in rabbits given native BSA. In chronic serum sickness, rabbits injected with a high dose (5 mg/rabbit/day), but not a low dose (500 μg/rabbit/day) of cationized BSA developed membranous nephropathy with severe proteinuria. In the group given cationized BSA, the levels and avidity of antibodies were lower than in the group given native BSA. Sucrose density gradient analysis of the complexes composed of ^{125}I-cationized BSA showed that IC formed *in vivo* were slightly larger than 7S. These antibody characteristics, i.e. low precipitation and low avidity, continued from early on to the late period of immunization. These results suggest that chemical cationization altered the immunogenicity of the antigen and resulted in the formation of antibody of low precipitability and low avidity, even during long-term immunization.

INTRODUCTION

Membranous nephropathy has been thought to result from two possible mechanisms: circulating IC deposition (Germuth, Flanagan & Montenegro, 1957; Dixon, Feldman & Vasquez, 1961) and *in situ* IC formation in the glomerular basement membrane (GBM) (Fleuren *et al.*, 1978; Couser & Salant, 1980; Border *et al.*, 1982). Recently, it has been established that antigenic charge is one of the factors determining the localization of IC (Couser & Salant, 1980) because of the presence of polyanion layer in the GBM (Kanwar & Farquhar, 1976). Using a perfusion system of experimental glomerulonephritis, Vogt *et al.* (1982) demonstrated that subepithelial deposits were induced by the injection of cationized antigen together with antibody against unmodified antigen, and the injection of IC composed of cationized antigens and antibodies directed against unmodified antigens can result in deposits along the peripheral capillary walls at both subepithelial and subendothelial sites (Gallo, Caulin-Glaser & Lamm, 1981; Koyama *et al.*, 1986). These observations indicated that even if there was no 'planted' cationic antigen in GBM, subepithelial deposits could be produced by injection of cationic antigen–antibody complexes formed *in vitro*. Caulin–Glaser, Gallo & Lamm (1983) and Lew, Tovey & Steward (1984b) also reported that subepithelial deposits could be induced by covalent cross-linked IC, which could not dissociate.

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Recent studies have shown that chemical cationization alters the interaction between antigen and antibody: the replacement of carboxyl groups of the antigen (BSA) to amines (amidation) altered the valence of antigen, and immune reaction between cationized antigen and antibody for unmodified, native antigen; avidity, precipitating efficiency and size of complexes decreased as the pI of antigen became higher (9·25–10·25 or over 10·25). There was no evidence by ultracentrifugation that IC composed of highly cationized antigens dissociated *in vitro* or *in vivo*, and the pI of the whole IC remained cationic (Koyama *et al.*, 1986).

From these observations, it seemed likely that in an active model of serum sickness induced by injection of cationized antigen, the animals might produce antibody of low avidity and low precipitating capacity, which could form small-sized soluble IC, especially if given a relatively high dose of antigen.

In this study, the characteristics of the antibodies and IC formed in acute accelerated and chronic serum sickness nephritis induced by cationized and native BSA in the rabbit are investigated and the possible role of cationization of antigen on the pathogenesis of membranous nephropathy is discussed.

MATERIALS AND METHODS

Preparation of antigens

Crystallized bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO) was used unmodified as native BSA and as substrate to prepare chemically modified cationic BSA. Chemical cationization (amidation) was carried out according to a modification of the method described by Hoare & Koshland (1967).

Characterization of antigens

The pI of each BSA was measured in thin layers of polyacrylamide gel, pH range 3·5–9·5 (Ampholine, Pageplate, LKB Instruments Inc., Rockville, MD), using an LKB flatbedded electrofocusing unit. The molecular size of cationized and native BSA was assessed by sucrose density gradient ultracentrifugation.

Radiolabelling

Native and cationized BSA were labelled with ^{125}I by the chloramine-T method.

Experimental design

New Zealand white rabbits weighing 3 kg were used throughout the experiments. The experimenal design is shown in Fig. 1. Acute serum sickness was induced by immunization of 4 mg of cationized and native BSA plus complete Freund's adjuvant, and at 14 days after the initial immunization 20 mg of each ^{125}I-labelled antigen were injected intravenously. Renal biopsies were performed before and at 15 min after the second injection. Blood was taken at 14 days before and at 15 min after the second injection for the analysis of antibody and IC. Chronic serum sickness was induced by injecting cationized and native BSA. At 14 days after the first immunization, 500 μg of either cationized or native antigen were injected intravenously every day for 6 weeks. At 9 weeks after the initial immunization, blood samples were taken for the analysis of antibodies and renal biopsies were performed to examine the histological findings at low dose antigen injection. At 10 weeks after the initial immunization, 5 mg of either cationized or native antigen were injected intravenously for 1 week. At 12 weeks after the first immunization, blood samples were obtained and renal biopsies were performed to examine the histological findings at high dose injection. To compare the difference in immunogenicity between cationized and native BSA, animals were immunized with 4 mg of each BSA in complete Freund's adjuvant and 5 mg of either cationized or native antigen were injected intravenously biweekly for 3 months to avoid the effect of repeated injections of antigens.

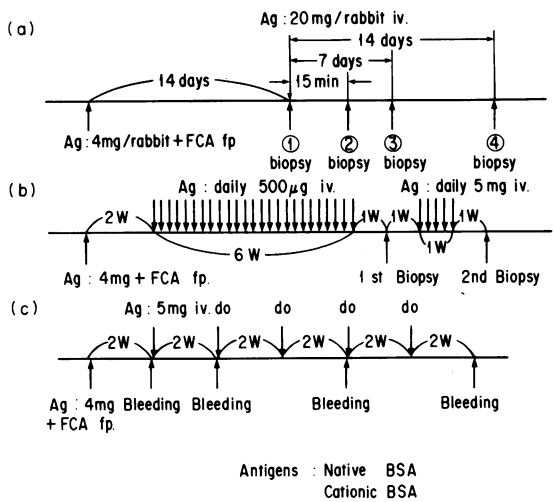

Figure 1. Experimental design: (a) acute accelerated serum sickness; (b) chronic serum sickness; (c) effect of cationization of antigen on antibody formation.

Characterization of antibodies

One ml of serum was dialysed against 0·02 M phosphate buffer (PB), pH 8·0, and then separated by DEAE cellulose column chromatography (0·02 M, PB, pH 8·0) and concentrated to 5 ml. Total antibody activity was measured as the antigen binding capacity-33 (ABC-33)*. Antibody avidity† was measured by the antigen dilution effect on ABC-33 (Minden & Farr, 1978). Precipitating antibodies and the valence of antigen (i.e. antigen/antibody molar ratio in great antibody excess) were calculated from the quantitative precipitation curve (Kabat & Mayer, 1961).

Size of antigen and IC

The sizes of antigens and IC were measured by sucrose density gradient ultracentrifugation (5–35% linear sucrose density gradient in PBS, pH 8·0). Antigens and IC were run at 100,000 *g* for 24 hr in a Beckman L-5 series Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) in an SW 27.1 rotor. BSA, human IgG and IgM were used as 4·6S, 7S and 19S markers, respectively. Fractions were collected from the bottom of the tube and radioactivity determined in an automatic gamma spectrometer.

Histological examination

The histological examinations were performed as described previously (Koyama *et al.*, 1978).

RESULTS

Characterization of antigens

The pI of cationized BSA was greater than 9·5, and that of native BSA was 4·5. The sizes of these antigens were almost the same, indicating that charge modification did not alter the molecular size of BSA. However, the valence of antigen ($\bar{x}=4{\cdot}5\pm1{\cdot}2$, $n=6$), calculated from the quantitative precipitation test in the animals given cationized antigen at 8 weeks after the immunization, was significantly lower than those given native BSA ($\bar{x}=7{\cdot}3\pm0{\cdot}6$, $n=7$) ($P<0{\cdot}001$).

*The ABC-33 value was calculated as follows:
ABC-33 = (ABC-33 end-point)** × (a)*** × (0·33) × (Ag)**** = μg ^{125}I-BSA bound/ml undiluted serum at the antigen concentration employed.

**ABC-33 end-point = the dilution of antiserum that would have precipitated exactly 33% of the antigen added.

***a = 1/a ml of the antiserum dilution used.

****Ag = the concentration of added ^{125}I-BSA.

†Antibody avidity was calculated using the following formula:
Avidity = value of ABC-33 at 0·01 μg ^{125}I-BSA/values of ABC-33 at 0·1 μg ^{125}I-BSA.

Table 1. Antigen-binding capacity-33, antibody avidity and precipitating antibody

Animals immunized with:	*n*	Precipitating antibody (μg/ml)	ABC-33 (μg BSA/ml)	Avidity
Cationized BSA	11	150·2±148·5	205·1±152·9	0·0406±0·016
Native BSA	4	596·8±271·5 $P<0·01$	1042·1±550 $P<0·02$	0·457±0·206 $P<0·001$

The quantitative precipitation test, ABC-33 and antibody avidity were measured 2 weeks after immunization (experimental protocol 1).

Table 2. ABC-33, antibody avidity and precipitating antibody levels for cationized BSA at 9 and 12 weeks after the first immunization in the animals with induced membranous nephropathy (experimental protocol 2)

	Precipitating antibody (μg/ml)	ABC-33 (μg BSA/ml)	Avidity
9 weeks $n=5$	161·9±55·5	176·7±72·5	0·35±0·13
12 weeks $n=5$	187·2±41·8	114·4±54·0	0·35±0·04

Effect of cationization on immune response of animals given cationized BSA

In the animals given cationized BSA, the ABC-33 values against cationized BSA at 14 days after the initial immunization were lower than those against native BSA in the group given native BSA. Antibody avidity and levels of precipitating antibodies against cationized BSA were also significantly lower than those against native BSA in the group immunized with native BSA (Table 1). In the animals in which membranous nephropathy could be induced, antibody avidity and the levels of precipitating antibody were also very low (Table 2). Although animals were immunized long-term in the group given cationized BSA, the values of ABC-33, precipitating antibodies and antibody avidity against cationized BSA were significantly lower than those against native BSA in the group given native BSA (Fig. 2).

The sizes of IC formed in the animals given cationized and native BSA in acute serum sickness

The sucrose density gradient ultracentrifugation studies showed that the sizes of IC in the animals immunized with cationic BSA were small, slightly larger than 7S after the second injection of ^{125}I-cationic BSA at 14 days (Fig. 3).

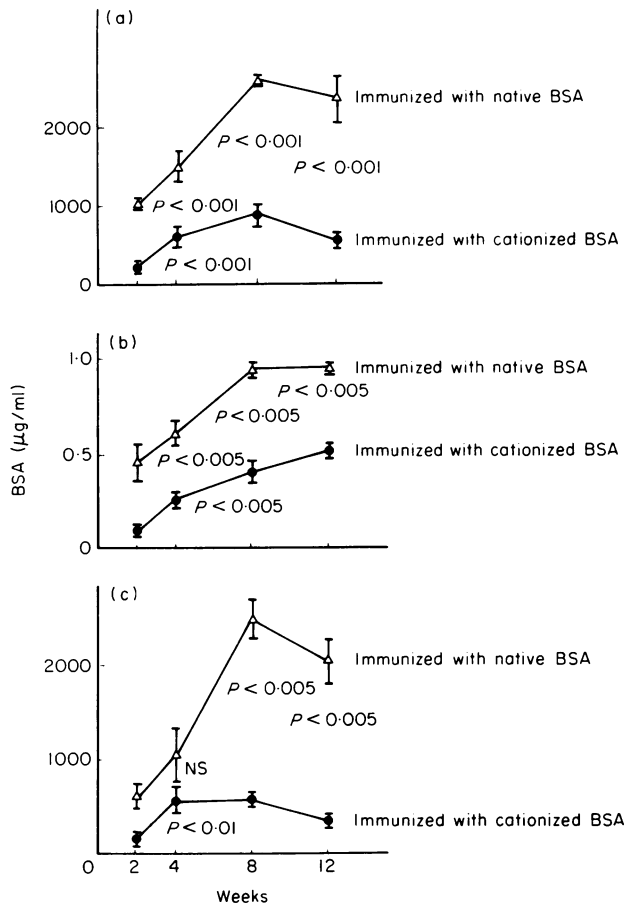

Figure 2. Effects of cationization of antigen on antibody formation. Changes in the values of (a) ABC-33, (b) antibody avidity and (c) precipitating antibodies during long-term immunization with cationized and native BSA (experimental protocol 3).

Histological findings

Acute experiment. In the group immunized with either native or cationized BSA at 14 days before the second injection of antigen, no fluorescence was observed in the glomerulus (Fig. 4a). At 15 min after challenge, diffuse deposition of rabbit IgG was observed along the peripheral capillary walls in the cationized BSA group (Fig. 4b). At 1 week after the challenge,

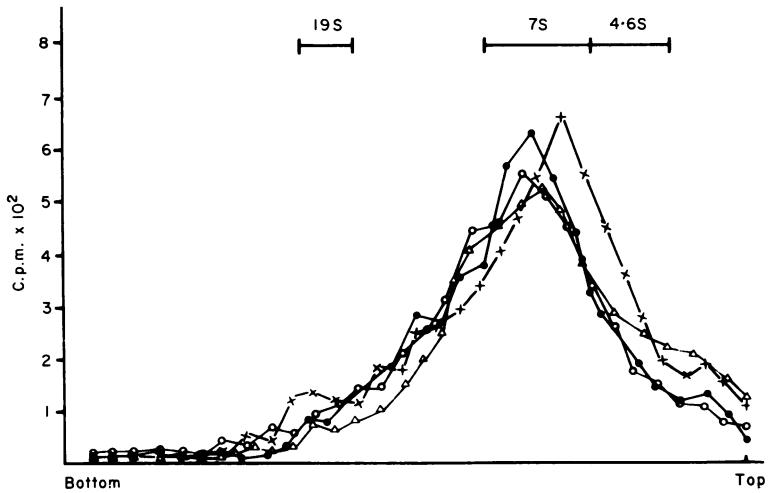

Figure 3. Distribution of antigens in the group given cationized BSA in sucrose density gradient analysis. Sera were taken at 15 min after the second injection of ^{125}I-cationized BSA in the four rabbits (●——●, △——△. ○——○, ×——×) that were induced acute serum sickness. The distribution of ^{125}I-cationized BSAs was observed at nearly or a little larger than 7S.

electron-dense, partly lucent deposits were seen in subepithelial sites on the GBM (Fig. 4c). On the other hand, in the animals given native BSA, at 14 days after the injection of native BSA, no or trace rabbit IgG was observed in the mesangium.

Chronic experiment. In chronic serum sickness, in the animals immunized with cationized BSA followed by the injections of low dose cationized BSA every day for 6 weeks, no or trace rabbit IgG was seen, but after the injection of high dose (5 mg/rabbit) of cationized BSA, diffuse peripheral granular localization of rabbit IgG and C3 were detected (Fig. 4d and e). Electron microscopic findings in this group showed that electron-dense deposits were seen at subepithelial sites on the GBM (Fig. 4f). In addition, light microscopic findings showed that 'spike' formation on the GBM was seen with moderate cell proliferation.

DISCUSSION

The mechanism of IC formation at subepithelial sites on the GBM using cationized antigen is now generally thought to be the result of *in situ* IC formation (Fleuren *et al.*, 1978; Couser & Salant, 1980). However, Gallo *et al.* (1981), Germuth *et al.* (1979), Lew, Staines & Steward (1984a) and we (Koyama *et al.*, 1986) demonstrated that in serum sickness of mice, IC preformed *in vitro* could induce subepithelial deposits, whether the antigen was cationized or native. The common feature for IC deposition at subepithelial sites on the GBM in passive serum sickness was IC composed of antibody of low precipitating capacity and low avidity. We have also shown that *in vitro* chemical cationization of the antigen (BSA) altered the antigen–antibody interaction; precipitating efficiency, antibody avidity and size of IC are reduced with increased pI. However, the IC composed of cationized antigen did not appear to dissociate *in vivo* or *in vitro*, and the pI of the whole IC remained cationic (Koyama *et al.*, 1986).

Kuriyama (1973) reported that membranous nephropathy could be induced by long-term immunization with native egg albumin (pI = 4·6), during which the animals produced antibodies of low precipitating capacity and low avidity. Therefore, we speculated that in active models of serum sickness using cationized antigens, as well as the formation of cationic IC with an affinity for GBM, the cationization would alter both antigenicity and the immune response. Thus, subepithelial deposits might be effectively induced by an antigen with these two characteristics.

In this study, we showed that in animals immunized with cationized antigen, the antibodies produced were indeed of lower precipitating capacity and lower avidity against cationized BSA, compared with those against native BSA in the animals given native BSA, throughout the period of immunization. These data confirmed our speculation and supported Kuriyama's report and our own previous data (Koyama *et al.*, 1978).

Examining the *in situ* IC formation theory, several questions arise. Many of the experiments on *in situ* IC formation were performed in systems involving perfused kidneys (Vogt *et al.*, 1982; Oite *et al.*, 1982) in which large amounts of cationized antigen were perfused into the renal artery, followed by antibody. However, one reservation about this procedure is that it is far from physiological. In intact animals, during the primary response of serum sickness, antigens could be planted after antibody formation had occurred. During secondary responses, in the presence of antibody, injected antigen could reach the polyanion layer of GBM without binding to antibody in the circulation.

In our experiments, during the primary response, neither antigen nor antibody was found in the glomerulus when antibody was formed. However, soon after the injection of cationized antigen, IC were observed in the glomerulus in a peripheral granular pattern. This observation indicated that the antigen did not fix long-term, compared with first phase of Masugi nephritis in which the injected 'antigen' (i.e. anti-GBM antibody) fixed to the GBM for a relatively long period of time. The sucrose density gradient study showed that cationized BSA bound to host IgG in the circulation 15 min after the second injection of cationized BSA.

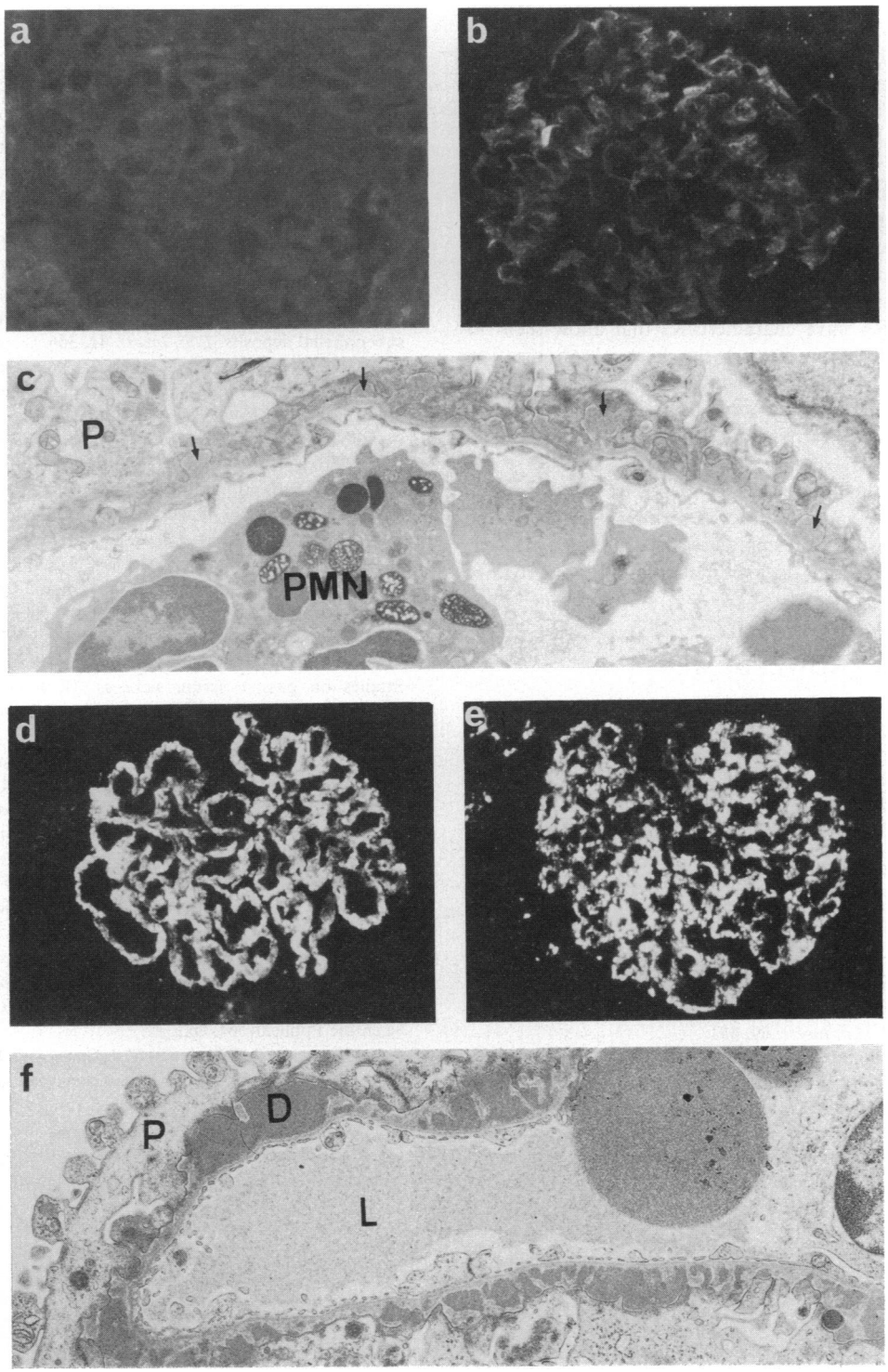

Figure 4. Histological findings in the group given cationized BSA. No immunofluorescence was seen before the second injection of antigen (a), whereas rabbit IgG was localized along the peripheral capillary walls soon after the second injection of antigen (b). Electron microscopic findings in the group given cationized BSA at 7 days after the second injection of cationized antigen (c). Electron-dense, partly lucent materials were seen on the subepithelial side of GBM. Immunofluorescence findings in the group immunized with cationic BSA at 12 weeks after the first immunization. Rabbit IgG (d) and C3 (e) were localized predominantly along the peripheral capillary walls. Electron microscopic findings in the group immunized cationic BSA at 12 weeks after the first immunization (f). Electron-dense deposits were seen on the subepithelial side of GBM. Magnification: (a), (b), (d) and (e) = 200; (c) and (f) = 8600.

In chronic serum sickness, when animals were given low doses (500 μg) of cationized BSA, and were therefore in antibody excess, neither deposition of rabbit IgG nor cell proliferation was observed. However, when the animals were given a relatively high dose of cationized BSA (5 mg), which produced equivalence or slight antigen excess (as calculated from the data in the quantitative precipitation test), intense diffuse peripheral granular localization of rabbit IgG and C3 was observed and severe proteinuria appeared. This suggests that even if cationized BSA is used, antigen excess is necessary to form subepithelial IC deposits, and this situation is also associated with the formation of small-sized IC *in vivo* and *in vitro*.

Our results suggest that IC formed in the animals immunized with cationized BSA have characteristics that allow them to bind to the GBM effectively, because the chemical cationization of antigen altered the immunogenicity of antigen and antigen–antibody interaction: replacement of carboxyl groups of antigen to amines caused low precipitating and low avidity antibody formation and formation of small-sized IC.

We conclude that subepithelial deposits following the injection of cationized antigen in active serum sickness were the result of the deposition of small-sized, cationically charged circulating IC composed of low precipitating, low avidity antibody.

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